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FINAL REPORT

**Diagnosis of Foot and Mouth Disease Virus
by Cloning, Sequencing and Monoclonal
Antibodies of VP1 of Israeli Serotypes**

Y. Becker, H. Yadin, D.M. Moore

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Appendix VI

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Title

Diagnosis of foot and mouth disease virus by cloning, sequencing
and monoclonal antibodies to VP-1 of Israeli serotypes

Investigators' Names (Principal listed first)

Prof. Y. Becker

Dr. H. Yadin

Dr. D. Moore

Investigators' Institutions

Hebrew University of Jerusalem

Kimron Veterinary Institute, Bet Dagan
Plum Island Animal Disease Center

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Yechiel Becker
Signature
Principal Investigator

Y. Haas
Signature
Institution's Authorizing Official
Professor Yehuda Haas
Chairman, Authority
for the Development

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c. Abstract

The major objectives of the research project were: a) the development of molecular tests for the diagnosis of FMDV, b) the study of the role of dendritic Langerhans cells in FMDV infection and immunization and c) development of a computer approach to the diagnoses of FMDV. The first objective was reached by initially developing specific nucleic acid probes to detect FMDV RNA and later by developing a rapid and accurate test based on the polymerase chain reaction (PCR). This test accurately detects viral RNA in clinical materials from infected animals in the field. The research on the second objective led to the demonstration that dendritic cells (LC) can be infected by FMDV. Since LC are involved in antigen presentation it was of importance that a mouse test for the efficiency of FMDV vaccines be developed. It was found that saponin, included in certain FMDV vaccines, is detrimental to LC in the skin of the immunized animals. The third objective was achieved by the development of computer methodology for the analysis of the viral VP1 polypeptide of different FMDV serotypes. This computer method provided diagnosis of the serotype of a new FMDV isolate. The computer analysis provided information on the antigenic domains in the structural proteins of FMDV isolates. Such computer analysis can be coupled with the PCR detection of a new FMDV isolate to provide a rapid diagnosis and analysis of the virus antigenicity. The active cooperation between members of the three collaborating laboratories made possible the successful conclusion of the project.

d. Objectives of the original proposal

Three major scientific objectives were set forward for the research on FMDV at the Kimron Veterinary Institute to attain its current molecular level. In addition, the research program included the training of an Israeli veterinarian at Plum Island Animal Disease Center in the molecular aspects of FMDV. This goal was achieved when the officials of KVI sent Dr. Oded Laor for one year of training in Dr. Moore's laboratory.

The three major scientific objectives were:

Objective 1. Development of molecular tools for the diagnosis of FMDV.

This objective was fully accomplished by the development of a rapid and accurate polymerase chain reaction (PCR) test for the diagnosis of FMDV RNA in clinical materials from FMD-suspected animals in the field. The use of magnetic beads covered with oligo dT for the isolation of FMDV poly A⁺ RNA allows rapid (5 min.) isolation of the viral RNA which can be amplified with the synthetic amplimers by the PCR method. Synthetic amplimers which amplify part of the conserved FMDV RNA polymerase gene provide the detection of all FMDV RNA from all serological types in the clinical material, while amplimers which allow the amplification of part of the VP1 nucleotide sequence allow differentiation between O1 and Asia FMDV isolates. The PCR method is currently used for the diagnosis of FMDV in recent outbreaks. The isolation of virus from the clinical materials in cell cultures (leading to accumulation of FMDV mutants with mutations in the VP1 sequence) is not needed for virus diagnosis since the PCR test is accurate and sensitive. The PCR test is superior to other molecular methods that we initially developed, like the FMDV riboprobes, to detect FMDV RNA. The cloning in bacterial plasmids of cDNA made from O1 GESHUR and Asia-1 Israeli isolates provided us with cDNA clones that

make possible the sequencing of VP1 and the synthesis of synthetic amplimers for specific serotype diagnosis. Such amplimers will allow detection of mutations in the VP1 gene in FMDV field isolates and the diagnosis of antigenic changes in FMDV field isolates.

Attempts to develop monoclonal antibodies to the Israeli FMDV isolates as well as the development of the Elisa test for FMDV diagnosis are still in progress. It is hoped that the sequencing of the VP1 cDNA will provide the antigenic properties of O1 GESHUR and Asia isolates to allow the synthesis of antigenic peptides for the production of monospecific polyclonal or monoclonal antibodies needed for perfecting the current diagnostic complement fixation test which is the acceptable test for FMDV diagnosis.

Objective 2: Role of skin Langerhans cells (LC) in bovine skin and in the immunization with FMDV vaccines.

The second objective of the project provided novel results on the role and function of LC in the immunization with FMDV vaccines and in the interaction with FMDV. Together with Dr. E. Sprecher (HU) and Dr. D. David (KVI) a practical test for the efficacy of commercial FMDV vaccines was developed. It was found that FMDV vaccines which contain saponin destroy LC after injection into the skin, thus affecting the cells which present FMDV antigens to the immune cells in the immunized animal.

Further studies on bovine skin LC allowed their purification by the magnetic beads covered with anti-Ia (mouse) monoclonal antibodies while LC were covered with mouse anti-bovine-Ia monoclonal antibodies. Such methods allowed studies on the interaction of FMDV with isolated bovine skin LC. In addition, a method to keep bovine LC under culture conditions without loss of viability was developed to allow future studies on the processing of FMDV antigens for presentation of T cells. Such studies are

of importance for the understanding of anti-FMDV immunity.

Objective 3: Development of a computer data bank of FMDV nucleotide sequences for the analysis of antigenic changes in FMDV isolates.

The third objective of the project provided a computer analysis of the VP1 amino acid sequences which can differentiate between FMDV serotypes. This computer method was put to use to distinguish between European and Iranian FMDV isolates. The computer analysis of antigenic domains in VP1 of different FMDV serotypes provides a novel computer method for assessment of the importance of mutations in VP1 of FMDV isolates in respect to antigenicity changes. This computer method will allow rapid analysis of the antigenicity in the VP1 and other capsid proteins of FMDV isolates diagnosed by the PCR test. A combination of PCR technology and the computer technology will make diagnosis of FMDV in infected animals rapid and accurate with the possibility of rapid decision regarding immunization and prevention of virus spread. The third objective of the program was achieved.

e. Body of the report

Objective 1: Molecular tests for rapid diagnosis of FMDV

I Utilization of the polymerase chain reaction (PCR) for FMDV cDNA synthesis and sequencing.

a. Usefulness of the PCR method

It is possible to amplify cellular and viral nucleic acids using oligonucleotide primers complementary to each of the two DNA strands of a DNA sample. When viral RNA is used synthesis of double-stranded cDNA is carried out prior to PCR. Repeated cycles of denaturation, primer annealing and extension of the DNA strands result in the exponential accumulation of the DNA sequence defined by the two primers. In a recent study, Torgersen et al (2) used the PCR method to amplify and characterize 380 bp of the VP1 nucleotide sequence of unidentified rhinoviruses isolated from patients. This study showed that rhinovirus RNA was reverse transcribed into cDNA which, with specific primers could be PCR amplified and the sequence of the amplified cDNA was directly determined. In view of the presence of low amounts of FMDV RNA in the isolated material (see attached reports by Dr. Moore and Laor), it seems that adaptation of the PCR method to detection and characterization of RNA from FMDV field isolates will be of great importance in the diagnosis of FMDV.

b. Synthetic oligonucleotides for FMDV VP1 PCR

In collaboration with H. Torgersen (Institute Biochemistry, Vienna, Austria) the following oligonucleotides were designed (Table 1) and synthesized (Dr. G. Darai, Heidelberg Univ. Heidelberg, FRG). The sequence of the oligonucleotides was derived from the nucleotide sequence of the VP1 in FMDV O₁K strain (3) which should resemble the sequence of the Israeli isolate O₁ GSHUR.

Table 1. Synthetic oligonucleotides for PCR on FMDV RNA

	3287 - 3304 (PCR1)
oligo 1	5' CAA CTG CTT ACC ACA AGG 3' (18 nucleotides)
	3694 - 3676 (cDNA) reverse transcription
oligo 2	5' AGT TCG ACC TAA CGT CGGA 3' (19 nucleotides) Taq I
	3637 - 3620 (PCR 2)
oligo 3	5' CCA ACT TGA GAA GGT CAA 3' (18 nucleotides)
	3487 - 3470 (sequencing primer)
oligo 4	5' TCG CTT TGA TGG CAC CGT 3' (18 nucleotides)

c. FMDV DNA and RNA for initial PCR experiments

Since FMDV RNA must be dealt with under strict security conditions, the preliminary experiments on the PCR technique will be done on the recombinant plasmid pGEM-5(z)-FMDV1034. This plasmid contains a 1034 bp DNA fragment flanked by T7 and SP6 promoters for RNA synthesis. In-vitro synthetic VP1 RNA will be used for the PCR experiments.

d. detection of FMDV RNA amplified by PCR. After the demonstration that the in vitro synthesised VP1 RNA can be amplified by PCR, experiments to develop the technology were done with RNA extracted from cells infected with O1 GESHUR isolates and on RNA from clinical materials. The results are presented in two manuscripts. 1) Detection of FMDV RNA amplified by PCR and 2) Detection of FMDV RNA in clinical material using the PCR reaction.

Detection of FMDV RNA Amplified by the Polymerase Chain Reaction (PCR)

Oded Laor^{1,2}, Helge Torgersen³, Hagai Yadin² and Yechiel Becker¹

1 Department of Molecular Virology
Faculty of Medicine
Hebrew University of Jerusalem
Jerusalem, Israel

2 Kimron Veterinary Institute
Beit Dagan ,Israel

3 Department of Biochemistry
University of Vienna
Vienna ,Austria

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SUMMARY

Molecular detection of Foot and Mouth Disease Virus (FMDV) using the Polymerase Chain Reaction (PCR) technique is a rapid and accurate method. In this study we present the PCR method for the detection of FMDV RNA in infected BHK cells. Using the PCR technique and two primers selected from the RNA Polymerase gene, a conserved sequence in all types and subtypes of FMDV, we were able to detect FMDV RNA present in RNA preparations extracted from the FMDV-infected cells. RNA from uninfected control cells gave negative results. Another set of primers selected from the nucleotide sequence of the variable VP1 gene enabled us to show variation in the PCR reaction among different FMDV Israeli isolates. Two O1 type FMDV isolates out of 6 FMDV field isolates (including O1 Geshur) gave a positive PCR reaction while two other O1 isolates and two ASIA isolates were detected by using the RNA Polymerase gene synthetic oligonucleotides but were not detectable using the VP1 synthetic oligonucleotides. Serial dilutions of the amount of RNA used in each reaction showed that a very small amount of RNA may be detected by the PCR technique. The PCR products from the RNA polymerase and the VP1 genes were sequenced and the nucleotide sequences obtained were compared to a known nucleotide sequence of the FMDV O1 genom.

INTRODUCTION

Foot and Mouth Disease (FMD) has a worldwide distribution and is of cardinal importance for agriculture. Although death rates of FMDV - infected animals may be low, losses due to reduction in milk and meat production should not be underestimated. In addition, FMDV epidemics require extensive vaccination of livestock and result in reduced export. Due to the rapid spread of FMDV and rapid mutation rate leading to a multiplicity of known serotypes and subtypes, (Cheung et al., 1983), confusion of the clinical picture of FMD with other virus diseases is eminent. The possibility of a carrier state among infected animals (Wittmann, 1990) makes diagnosis, as well as control, quite difficult. The adoption of rapid and accurate diagnostic procedures to detect and identify FMDV in carrier animals is Essential to an effective FMD control program.

The current availability of the Polymerase Chain Reaction (PCR) Technique (Saiki et al., 1988) allowed the development of a rapid and accurate method to identify FMDV RNA in RNA preparations from infected cells.

MATERIALS AND METHODS

Amplification of FMDV VP1 sequence by the PCR Reaction

Total RNA from the Israeli FMDV isolate 01 Geshur (01G) was used as a template for PCR amplification. The virus was grown in BHK cell culture and total RNA was isolated by the acid guanidinium thiocyanate method. As a positive control we used a recombinant plasmid with an insert of 1034 bases from the VP1 area of the FMDV Type 01 Kaufbeurene (01K) (Kurz et al., 1984). The insert, cloned in a pBR 322 plasmid, was provided by Prof. H. Schaller (Heidelberg University) and was

transferred to pGEM 52 plasmid in our laboratory. In vitro transcription of the recombinant 01K 1034 in the pGEM 52 plasmid was carried out in order to obtain virus-specific RNA as a positive control. The plasmid was cut with the restriction enzyme PstI (Boehringer Mannheim) and a Stratagene RNA transcription Kit was used. Primers were selected for the VP1 sequence, based on the reported VP1 sequence of FMDV 01K.

The direct primer: AGTTCGACCTAACGTCGGA (Oligo # 2), a 19-mer oligonucleotide, is located in the P12 area adjacent to the 3' end of the VP1 (supplied at concentration of 100 pmol/ μ l)

The reverse primer: CAACTGCTTACCACAAAAGG (Oligo # 1), a 20-mer oligonucleotide, is located 424 bases upstream from the direct primer, within the VP1 area (supplied at a concentration of 100pmol/ μ l).

The direct primer (Oligo # 2) was also used as the reverse transcription primer.

1 μ l of the total RNA of 01G (at a concentration of 3.5 μ g/ μ l) and 1 μ l of the in vitro-transcribed (1034 01K) RNA were reverse transcribed to obtain cDNA for the PCR reaction.

The reaction volume was adjusted to 11 μ l with ddH₂O and incubated for 5 minutes at 70°C.

To each sample the following was added:

1 μ l RNasin (40 U/ μ l - Promega)

1 μ l dNTPs (10 mM Pharmacia)

1 μ l AMV Reverse transcriptase (16.3 U/ μ l, Life Science Inc.)

2 μ l 10X buffer (according to the manufacturer's recommendation)

4 μ l ddH₂O

The total reaction volume of 20 μ l was incubated for 1 h at 42°C.

dNTPs were supplied at 100 mM each and were mixed to obtain a final concentration of 10 mM each.

10 μ l of each cDNA obtained was directly forwarded to PCR reaction.

RNA samples used for the PCR reaction:

1. O1G total RNA at a concentration of 3.5 µg/µl was first reverse transcribed (as described above) and used for the PCR reaction.
2. RNA transcribed from the recombinant plasmid 1034 O1K was reverse transcribed and used for PCR reaction.
3. DNA recombinant plasmid 1034 O1K (concentration 1 ng/µl) was used as a positive control for the PCR reaction. 1 µl was taken for the reaction and the volume was brought to 10 µl by the addition of dd H₂O.
4. Negative control (no RNA or DNA introduced to the reaction) 10 µl of dd H₂O was used.

For samples 1 and 2, where 10 µl of the reverse transcribed reaction were directly followed by PCR, the following reagents were added to bring volume reaction to 50 µl.

1 µl oligo 2

1 µl oligo 1

1 µl 2M KCl

1 µl Taq DNA Polymerase (4 U/µl - IBI)

36 µl dd H₂O

In samples 3 and 4 the 50 µl reaction volume was composed of :

10 µl DNA sample

1 µl oligo 2

1 µl oligo 1

1 µl dNTPs 10 mM (Farmacia)

5 µl 10X amplification buffer (supplied with the enzyme)

1 µl Taq DNA Polymerase (4 U/µl)

31 µl dd H₂O

Two drops of mineral oil were placed on top of each reaction solution to prevent evaporation.

All 4 samples were placed in a HYBAID Thermal Reactor for 35 cycles.

Denaturation at 93°C for 30 seconds

Annealing at 55°C for 30 seconds

Polymerization at 72°C for 1 minute

The third step was increased to 10 minutes at the end of the reaction.

10 µl of each sample were run on a 1.6% Agarose gel.

Amplification of O1 Geshur VP1 with modified primers

For this experiment a new primer was selected.

oligo # 5 ; 5' TTCGAAACGGCACGGTCGGA 3' based on the known sequence of O1G. Oligo 5 replaced the primer oligo 1.

The primer used to reverse transcribe the O1G RNA was oligo 2 as described above.

1 µl of the cDNA obtained was used for PCR reaction using oligo 2 and oligo 5 as primers. 50 µl reaction volume contained 1µl cDNA, 100 pmol of each of the primers, 10 mM Tris HCl PH 8.3, 50 mM KCl, 1.5 mM MgCl₂, dNTPs (Pharmacia) 10 mM each and 4 units Taq DNA Polymerase (IBI)

Samples were placed on Programmable Thermal Controller

(MJ Research Inc) for 35 cycles composed of :

Denaturation at 93°C, 30 seconds

Annealing at 55°C, 30 seconds

Polymerization at 72°C 1 minute

The last step was increased to 10 minutes at the end of the reaction.

10 µl of each sample were electrophoresed on a 1.6% agarose gel.

(data not shown)

Amplification of VP1 sequence of six different FMDV Israeli isolates

The same amplimers (Oligo 2 and Oligo 5) were used to test the variability in the VP1 sequence of six Israeli isolates : O1 GESHUR, O1 DALTON, O1 GAZIT, O1 SHCHEM, ASIA 1 and ASIA subtype SHAMIR.

All these FMDV isolates were grown in BHK cells and RNA was extracted by the Acid Guanidinium Thiocyanate method. PCR reaction was made as described above.

Amplification of FMDV RNA Polymerase sequence in the RNA of six Israeli isolates.

The RNA Polymerase gene is a conserved nucleotide sequence present in all reported FMDV genomic sequence. Using the computer program of the University of Wisconsin Genetic Computer Group (UWGCG) (Devereux et al., 1984), we compared the published FMDV genomes O1K (Forss et al., 1984), A12 (Robertson et al., 1985) and A10 (Boothroyd et al., 1981) and related amplimers that can be used to detect all FMD types and subtypes.

The Oligonucleotides that were selected are:

Direct primer oligo # 7 : 5' GTAAAGTGATCTGTAGCTTGG 3'

Reverse primer oligo # 9 : 5'TTCGAGAACGGCACGGTCGGA 3'

Both amplimers amplify a 1 Kb segment within the RNA polymerase gene.

1 µl RNA of each of the six FMDV subtypes was incubated with 100 pmol primer oligo 7 at 70°C for 5 minutes in 11 µl reaction volume.

The annealed RNA/Primers were reverse transcribed with AMV Reverse Transcriptase as described above.

1 µl of the cDNA obtained was amplified in a PCR reaction as described for the second experiment in this paragraph.

10 µl of the amplified sample were run on 1.6% agarose gel.

Serial dilutions of FMDV RNA

Three dilutions, 1:10, 1:100 and 1:1000, were made from the total RNA extracted from BHK cells infected with FMDV isolate O1G. cDNA was synthesized, using oligo 7 as primer, from 1µl of each of the dilutions. 1µl of each of the cDNA obtained was forwarded to the PCR reaction and 1Kb segment within the RNA polymerase gene was amplified using the amplimers oligo 7 and oligo 9 as already described.

Purification and sequencing of the PCR products

The amplified segment of the VP1 gene (424 bases long) and that of the RNA polymerase gene (1 Kb long) were run on a low gelling temperature agarose gel. The desired segments selected according to their sizes were removed and cDNA was extracted from the gel as previously described by Wieslander, (1979).

The purified segments were sequenced by the dideoxy-mediated chain termination method (Sanger et. al., 1977) using the "PUC sequencing kit" for DNA sequencing (Boehringer Mannheim).

Computer program

The "bestfit" computer program was used (Devereux et al., 1984).

RESULTS

The nucleotide sequence of FMDV RNA Polymerase was chosen for amplification since it is a conserved sequence in the FMDV genome of all FMDV types. Comparison of the published nucleotide sequences of several FMDV isolates using the "Best fit" program in the University of Wisconsin Genetic Computer Group (UWGCG) software (Devereux et al., 1984) showed a marked similarity among the sequences of all FMDV types. We selected two oligonucleotide sequences limiting 1 Kb segment, (designated oligo 7 and oligo 9) to amplify RNA from cells infected with six Israeli isolates. Successful amplification by the PCR reaction using oligo 7 and oligo 9 as primers is shown in Fig. 1. PCR reaction using the VP1 amplimers (oligo 1 and oligo 2) as primers is shown in Fig. 2. The oligonucleotides were selected according to the reported sequences of O1K isolate (Kurz et al., 1984).

The in vitro-transcribed RNA from the chosen VP1 gene of O1K and O1 Geshur RNA as well as cloned VP1 DNA present in the recombinant plasmid 1034 O1K (containing 1034 nucleotides) were amplified.

Although not measurable, a lower yield of amplified product, based upon the amplified cDNA content in the bands, was observed in O1G VP1 sequence as compared to the in vitro-transcribed O1K RNA. Examination of data provided on the VP1 sequence of O1G (Pfaff et al., 1988) revealed a difference, relative to O1K, of two nucleotides in the nucleotide sequence used for the synthesis of Oligo 1 amplimer.

A new primer was synthesized based on the O1G nucleotide sequence (designated oligo 5). O1G RNA was successfully amplified using oligo 2 and oligo 5 as primers. (data not shown). PCR reaction was carried out with total RNA purified from infected BHK 21 cells with each culture infected with one of six different Israeli FMDV isolates. Four of these isolates were serologically diagnosed as FMDV type O1, and two others as FMDV ASIA. For this reaction the primers used were oligo 2 and oligo 5.

A clear positive result was observed with the O1 Geshur RNA sample, O1 Gazit gave a slightly positive reaction and all other samples were negative.(Fig. 3)

Serological data indicated marked similarity between O1 Geshur and O1 Gazit compared to the other O1 Israeli isolates. Molecular analysis (Samuel et al., 1990) showed that O1 Geshur and O1 Dalton are not closely related.

Serial dilutions, 1:10, 1:100 and 1:1000, were made from the total RNA extracted from BHK cells infected with O1G isolate. Undiluted RNA and dilutions 1:10 and 1:100 were successfully amplified with the PCR reaction and with respectively reduced intensity. The 1:1000 dilution was negative (Fig. 4)

The PCR amplified segments of the VP1 and the RNA polymerase genes were purified on a low gelling temperature agarose gel and directly sequenced. A segment of 177 bases long was obtained from the RNA polymerase gene (fig. 5 A) and a 159 bases long sequence was obtained from the amplified VP1 area (Fig. 5 B). The sequences were compared to the known sequence of FMDV type O1K (Foss et.al., 1984) using the "bestfit" program in the UWGCG software (Devereux et. al., 1984) in order to verify that the amplified polynucleoyide segments were the correct ones. High similarity, over 97% , was observed between the RNA polymerase sequence compared (Fig. 6 A). A lower percentage of similarity yet relatively high, over 86% , was observed when the amplified VP1 sequence was compared to the VP1 sequence of O1K. (Fig. 6 B).

This result leaves no doubt that the amplified segments are sequences of the FMDV genome.

DISCUSSION

The use of the PCR technique allowed us to develop a rapid and accurate method to detect FMDV positive sense RNA extracted from cells infected with Israeli FMDV isolates. This method enabled us to detect FMDV RNA even when present in very small amounts. For the detection of FMDV RNA in the infected cells, it was necessary to use nucleotide sequences from the viral genome that are conserved in all known FMDV types and subtypes. Using the "Best fit" program in the UWGCG software, it was possible to select two primers, designated oligo 7 and oligo 9. All Israeli FMDV isolates propagated in BHK cells and their RNA examined by the PCR technique using the primers from the RNA polymerase sequence gave positive results. However not all of the Israeli FMDV O1 subtypes were detectable with the primers for the detection of O1 VP1 sequence suggesting the presence of mutations in the VP1 nucleotide sequence in some Israeli isolates.

The use of the VP1 sequence amplimers (oligo 2 and oligo 5), which were synthesized on the basis of the sequence of O1G (Pfaff et Al., 1988), indicated that differences exist in FMDV isolates in the nucleotide sequence of the VP1 gene that codes for the immunogenic domains in the virion capsid. From the four FMDV isolates that were serologically diagnosed as FMDV type O1, two isolates reacted positively in the PCR reaction, though with different levels of cDNA synthesis. No amplification by the PCR was found in the other two FMDV isolates. These results showed that the VP1 sequence of the two FMDV isolates, O1G and O1 Gazit, resembled each other, while the other two did not. Two other FMDV isolates not detected by the PCR technique with the VP1 amplimers were serologically diagnosed as ASIA subtype. These results suggest that amplimers oligo 1, 2 and 5 are useful in the detection of O1 FMDV isolates. Direct sequencing of the VP1 gene of all the known Israeli isolates including the VP1 of ASIA isolates

will allow the selection of specific amplimers from the VP1 gene coding for the antigenic loop in VP1 protein. The amplimers from the RNA Polymerase sequence will serve to detect whether FMDV RNA is present in the suspected pathological material. The FMDV-specific VP1 amplimers will enable the rapid diagnosis of the type and subtype of the FMDV isolate prior to sequencing of the PCR cDNA product. Direct determination of the nucleotide sequence from the VP1 gene will provide information on the structure of the antigenic domain in the VP1 polypeptide using computer program (Becker, 1987 ; Becker, in preparation).

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Fig. 1 PCR of FMDV RNA using amplimers from the RNA Polymerase gene sequence.

1. 01 Geshur - 1Kb segment amplified from the RNA Polymerase area using oligo 7 and oligo 9 as primers.
2. 01 Dalton
3. 01 Gazit
4. 01 Shchem
5. ASIA 1
6. ASIA Shamir
7. Negative control (no RNA or DNA in the reaction)
8. DNA molecular weight size Marker No. VI

Fig. 2 PCR analysis of FMDV using the amplimers oligo 1 and oligo 2 within and near the VP1 gene sequence.

1. In vitro-transcribed RNA 1034 01K - as positive control for the reverse transcription reaction.
2. Total RNA 01 Geshur
3. DNA molecular weight size marker No. VI
4. DNA recombinant plasmid 1034 01K - as positive control for the PCR reaction.
5. Negative control - no DNA or RNA in the system.

Fig. 3 PCR analysis of local O1 and ASIA FMDV isolates using the amplimers for the VP1 gene sequence.

1. O1 Geshur - Positive result with the primers oligo 2 and oligo 5 for VP1 area.
2. O1 Geshur - negative result with the primers oligo 2 and oligo 5 for the VP1 area.
3. O1 Dalton - negative result using oligo 2 and oligo 5 as primers.
4. ASIA Shamir - negative result using oligo 2 and oligo 5 as primers.
5. O1 Gazit - Slight positive result with oligo 2 and oligo 5 as primers.
6. O1 Scheme - negative result with oligo 2 and oligo 5 as primers.
7. ASIA 1 - negative result using oligo 2 and oligo 5 as primers.
8. DNA molecular weight size marker No. VI
9. Negative control - no DNA or RNA in the system.

Fig. 4 Serial dilutions of total RNA extracted from BHK cells infected with O1G isolate, amplified with the PCR technique using the amplimers oligo 7 and oligo 9, within the RNA Polymerase gene.

1. Undiluted O1G total RNA - 1Kb segment amplified.
2. Dilution 1:10
3. Dilution 1:100
4. Dilution 1:1000
5. Negative control (no RNA or DNA in the reaction)
6. DNA molecular weight size marker No. VI

A

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1  TCCAGCTGAC AAAAGCGACA AAGGTTTTGT TCTTGGTCAC TCCATTACCG
51 ATGTCACTGT TCATCAAAAG ACACTTCCAC ATGGACTATG GAACTGGGTT
101 TTACAAACCT GTGGCTCTCG AAGACCCTTG AGGCCTATCC TCTCCTTTGC
151 ACGCCGTGGG ACCATACAGG AGAAGTT

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B

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1  CGCTGCCCAC CTCCTTCAAC TACGGTGCCA TCAAAGCTAC TCGGTGACCG
51 AGCTGCTTTA CCGCATGAAG AGGGCTGAGA CATACTGTCC GACGCCTCTA
101 TTGGCCATTC ACCCAAGTGA AGCTAGACAC AAGCAGAAAA TAGTGGCACC
151 TGTGAAACA

```

Fig. 5 Direct nucleotide sequence of the PCR product.

The amplified DNA was purified on a low gelling temperature agarose gel and sequenced using the PUC sequencing kit for DNA sequencing (Boehringer Mannheim)

A. RNA polymerase gene, 177 bases long sequence.

B. VP1 area, 159 bases long sequence.


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A 7408 TCCAGCTGACAAAAGCGACAAAGGTTTGTTCCTTGGTCACTCCATTACCG 7457
      |||
      1 TCCAGCTGACAAAAGCGACAAAGGTTTGTTCCTTGGTCACTCCATTACCG 50
7458 ATGTCACT.TTCCTCAAAAGGCACTTCCACATGGACTATGGAAGTGGGTT 7506
      |||
      51 ATGTCACTGTTTCATCAAAAGACACTTCCACATGGACTATGGAAGTGGGTT 100
7507 TTACAAACCTGTGATGGCCTCAAAGACCCTTGAGG.CTATCCTCTCCTTT 7555
      |||
      101 TTACAAACCTGTG..GCTCTCGAAGACCCTTGAGGCCTATCCTCTCCTTT 148
7556 GCACGCCGTGGGACCATACAGGAGAAGTT 7584
      |||
      149 GCACGCCGTGGGACCATACAGGAGAAGTT 177

      Gap Weight: 5.000      Average Match: 1.000
      Length Weight: 0.300      Average Mismatch: -0.900

      Quality: 149.3      Length: 179
      Ratio: 0.844      Gaps: 3
      Percent Similarity: 97.143      Percent Identity: 97.143

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Fig.6 Comparison of the amplified RNA polymerase and the VP1 sequences of FMDV type 01G to the known sequence of the FMDV type 01K, using the "bestfit" program of the UWGCG software.

B. Comparison of the VP1 sequence of FMDV type 01K to a 159 bases long sequence out of the amplified DNA from the VP1 of FMDV type 01G by the PCR technique using specific amplimers.

**Detection of FMDV RNA in clinical material
using the PCR reaction on poly A RNA isolated
with oligo dT on magnetic beads**

Oded Laor^{1,2}, Hagai Yadin², Dalia Hai² and Yechiel Becker¹

**1 Department of Molecular Virology
Faculty of Medicine
Hebrew University of Jerusalem
Jerusalem Israel**

**2 FMDV Unit, Department of Virology
Kimron Veterinary Institute
Beit Dagan, Israel**

SUMMARY

Four FMDV samples from suspected animals were received for diagnosis. In addition to the routine serological tests, injection into baby mice, inoculation of pig kidney (PK) cell culture and complement fixation test, we used a molecular diagnostic tool, the polymerase chain reaction (PCR) for the detection of FMDV genomic RNA. The RNA was extracted from the clinical material (tongue epithelium or skin epithelium) using the DYNABEADS oligo (dT)25 which selectively remove the polyadenylated RNA. The RNA thus extracted was released from the beads and was used in the PCR reaction after cDNA synthesis using primers selected to amplify a 1 Kb size segment present in the conserved gene of the viral RNA Polymerase. Another set of amplimers localized in the VP1 sequence was selected and used to synthesize a 424 Kb segment according to the known nucleotide sequence of the Israeli FMDV isolate 01 Geshur.

The presence of FMDV RNA in the clinical material and its relation to the 01 type was determined in three samples while the fourth clinical sample gave negative results in virus isolation and Complement Fixation tests carried out on these clinical samples confirmed the PCR results.

INTRODUCTION

Foot and Mouth Disease (FMD) has a worldwide distribution and is of cardinal importance for agriculture. Due to the rapid spread of FMDV and the rapid mutation rate leading to multiplicity of serotypes and subtypes, over 70 known subtypes (Cheung et al., 1983), the confusion of FMD's clinical picture with other diseases is eminent. The possibility for carrier state among infected animals (Wittmann, 1990) makes diagnosis as well as control quite difficult. Essential to an effective FMD control program would be the adoption of rapid and accurate molecular diagnostic procedures, based on the use of RNA extracted directly from clinical material from suspected animals in the field, without the need of passing the virus in cell cultures. The current availability of the Polymerase Chain Reaction (PCR) technique (Saiki et al., 1988) allowed us to develop a rapid and accurate method to identify FMDV RNA in RNA extracted from clinical material.

Preparation of Viral FMDV RNA from virions requires their concentration with polyethyleneglycol (PEG) and purification of the virus on a sucrose or cesium chloride gradients. The RNA is then extracted with phenol/chloroform and precipitated with ethanol as already described (Beck and Strohmair, 1987). Isolation of mRNA involves isolation of total RNA followed by selection of poly A (+) RNA by affinity chromatography on dT cellulose column (Aviv and Leder, 1972).

The genome of foot and mouth disease virus (FMDV) is a positive (+) sense polyadenylated RNA at its 3' end (Grubman et al., 1979). In order to isolate FMDV RNA we used a novel method designed for easy and rapid purification of poly A (+) sense RNA directly from tissues or cell cultures using DYNABEADS oligo (dT)₂₅ (Jakobson et al., 1990). This method is based on magnetic separation technology. Dynabeads oligo (dT)₂₅ are uniform, supermagnetic polystyrene beads 2.8 µm in diameter and contain a 25

nucleotide long chain of deoxythymidylate covalently attached to the bead surface via a 5' linker group. The separation of poly A (+) RNA relies on base pairing between the poly A at the 3' end of the RNA which forms hydrogen bonds with the oligo dT residues coupled to the surface of the beads which can be removed from tissue homogenate by a magnet.

The poly A (+) RNA prepared by this method directly from FMDV infected tissues can be forwarded without further purification for cDNA synthesis followed by PCR amplification (Saiki et al, 1988). In the present study this method was used for the detection of FMDV RNA in clinical samples from animals in the field.

MATERIALS and METHODS

Biological materials suspected for FMDV

Four samples from infected animals were examined :

- A. Tongue epithelial tissue from a cow showing clinical signs of FMD.
- B. Tongue epithelial tissue from a cow showing clinical signs from a second outbreak.
- C. Foot epithelial tissue from a sheep showing clinical signs from the same outbreak as sample B.
- D. Tongue epithelial tissue from a one-week-old lamb from a flock where 20 out of 150 lambs died.

Purification of RNA from sample A

The tongue epithelial tissue was washed twice with ice cold PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM $\text{Na}_4\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.4 mM KH_2PO_4), and then ground with the ultra turrax type T-25 (Janke and Kunkel). A small amount

of the ground tissue was transferred into two different eppendorf tubes spant to pellet tissue.

Extraction of RNA using the Dynabeads oligo (dT)25.

Method 1. One ml of lysis/binding buffer (100 mM tris-HCl pH 8, 500 mM LiCl, 10 mM EDTA, pH 8, 1% SDS 5, mM DTT) and 80 units RNasin (Promega) were added to the tissue pellet. After mixing, the solution was left at room temperature for 2 minutes. The lysate was centrifuged for 30 seconds in an eppendorf centrifuge and the supernatant transferred to an eppendorf tube containing 1.5 mg Dynabeads oligo (dT)25 suspended in 100 µl lysis/ binding buffer. The mixture was put on ice for 5 minutes to allow the poly A RNA to anneal to the oligo dT on the beads. After annealing the tube was placed on MPC-E Magnetic Particle Concentrator for 30 seconds and the supernatant was removed. The beads were washed twice with 500 µl Washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1mM EDTA, 0.1-0.3% SDS). The washed beads were incubated with 10 µl Elution buffer (2 mM EDTA pH 7.5) at 65°C for 2 minutes. The tube was placed on MPC-E and the eluted poly A RNA solution then removed.

Method 2. The tissue pellet was resuspended in 100 µl Lysis buffer (10 mM Tris-HCl pH 7.5, 0.14 M NaCl, 5 mM KCl, 1% Triton X 100) and 80 units of RNasin (Promega). The solution was placed on ice for 1 minute, centrifuged for 30 seconds and the supernatant was transferred to a new eppendorf tube containing 450 µg Dynabeads oligo (dT)25 suspended in 100 µl 2X Binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA, 0.4-1% SDS). The Solution was left on ice for 5 minutes to allow annealing of the oligo dT to the poly A (+) RNA . After annealing, the tube was placed on MPC-E for 30 seconds and the supernatant removed. The beads were washed twice with 200 µl Washing buffer. The washed beads were incubated with 10 µl Elution buffer for 2 minutes at 65°C. The tube was replaced on

the MPC-E for 30 seconds and the eluted poly A (+) RNA collected.

RNA purification from samples B and C

Each sample was treated separately. Approximately 1 gram of each of the suspected tissue was washed a few times with PBS. The washed tissue was transferred to a mortar, 1 ml of Lysis/Binding buffer, 80 units of RNasin and powdered glass were added and the tissue was ground in a mortar with a pestle. The ground tissue was centrifuged for 30 seconds in an eppendorf centrifuge and the supernatant transferred to an eppendorf tube containing 1 mg Dynabeads oligo (dT)25 suspended in 100 μ l

Lysis/Binding buffer. The mixture was put on ice for 5 minutes to allow annealing of the beads to the polyadenylated RNA. The beads were separated on the MPE-C, washed twice with 0.5 ml Washing buffer and then the poly A RNA was eluted in 10 μ l Elution buffer for 2 minutes at 65°C. Beads were removed with the MPE-C.

RNA purification from sample D

The tongue epithelial tissue was chopped and washed with PBS solution. Two trypsinizations were carried out using 0.5% trypsin in a total volume of 50 ml for 30 minutes each. Solutions were collected by filtration through a gauze and centrifuged 2000 RPM for 5 minutes to pellet cells. The trypsinized cells were resuspended in 0.5 ml PBS and counted. A total amount of 1.6×10^6 cells was obtained and the cells were used for RNA extraction with Dynabeads oligo (dT)25. Poly A RNA was extracted as described for the second extraction method in sample A.

Synthesis of cDNA and PCR

Each of the samples of purified poly A (+) RNA were reverse transcribed using AMV reverse transcriptase (Life Science Inc.) (Sambrook et al, 1989). A 19 mer synthetic oligonucleotide, located in the P12 area adjacent to the 3' end of VP1, (oligo # 2 ; 5' AGTTCGACCTAACGTCGGA 3') was used as a primer for the VP1 region. A 21 mer (oligo # 7 5' GTAAAGTGATCTGTAGCTTGG 3') located within the RNA Polymerase area was used to amplify this region.

One hundred pmol primer were annealed to 2 µl purified poly A (+) RNA at 70°C for 5 minutes in a reaction volume of 11 µl. The annealed RNA/Primer was incubated with 18 units of AMV reverse transcriptase and dNTPs (Pharmacia), 10 mM each, at 42°C for one hour in 50 mM Tris-HCl pH 8.3, 8 mM MgCl₂, 40 mM KCl and 40 units of RNasin (Promega) in a total reaction volume of 20 µl.

Five µl of the cDNA obtained were used in the PCR reaction. For the VP1 area the direct primer used was oligo # 2 (the one used for reverse transcription) and the reverse primer was a 20 mer oligonucleotide (oligo # 5: 5' AGTTCGACCTAACGTCGGA 3') located 424 nucleotides upstream from the direct primer. For the RNA polymerase gene amplification the direct primer used was oligo # 7 (used for reverse transcription) and the reverse primer was a 21 mer (oligo # 9: 5' TTCGAGAACGGCACGGTCGGA 3') located approximately 1 Kb upstream from the direct primer within the RNA Polymerase gene.

50 µl reaction volume contained 5 µl cDNA, 100 pmol of each of the primers, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, dNTPs (Pharmacia) 10 mM each and 4 units Taq DNA Polymerase (International Biotechnology Incorporation, IBI).

Samples were placed on a programmable thermal controller (M J Research)

for 35 cycles consisting of :

Denaturation 93°C 30 seconds

Annealing 55°C 30 seconds

Polymerazation 72°C 1 minute.

The last step was increased to 10 minutes at the end of the reaction. 10 µl of each of the reaction products was run on an 1.6% agarose gel.

Serological tests

Each clinical material was washed with PBS and ground with powdered glass in mortar and pestle in persence of Earle's Medium. The homogenate was centrifuged and the supernatant was taken for injection of 10 baby mice, inoculation of pig kidney (PK) cell cultures and for complement fixation test (CF)(Lombard et al., 1982). The CF test was carried out against the following antisera:

A. 01 BFS - British isolate, 1967

B. 01 GESHUR - Israeli isolate, 1985

C. 01 MANISA - Turkish isolate, 1969

D. 01 DALTON - Israeli isolate, 1988

E. A22 - Iraqi isolate, 1964

F. ASIA - Lebanese isolate, 1983

Supernatant recovered from sample C was too little for all tests, therefore, first PK cell culture was inoculated and CF test was carried out with the cell culture medium following cytopatogenic effect in the cell culture (CPE).

Sample B was anti complementary with the field clinical material therefore CF test was carried out with cell culture medium following CPE in the cells.

RESULTS

Four field samples of epithelial tissue from FMD suspected animals were examined using a novel method for RNA extraction (DYNABEADS oligo (dT)₂₅) and the PCR technique.

We were able to detect the presence of FMDV RNA in three out of the four samples. Sample A was obtained from a cow showing clinical signs (Fig. 1). The RNA was extracted using two slightly different methods. The PCR reaction was the amplimers for the detection of the RNA polymerase gene (oligo # 7 and oligo # 9) gave a positive amplification reaction although with different intensities (Fig.1 lanes 1 & 2). Specific amplimers for the VP1 nucleotide sequence derived from the sequence of FMDV isolate 01 Geshur, gave positive results though with a reduced intensity (Fig. 1 lanes 3 & 4). This result indicates that the examined FMDV RNA could be related to the FMDV type 01. Samples B (Fig. 2) and sample C (Fig. 3) obtained from a cow and a sheep, respectively, both showing clinical signs from the same outbreak, gave clear positive results using the amplimers for the RNA polymerase gene (Fig.2 lane 1 & Fig.3 lane 1). The amplimers for the VP1 region also gave positive results (Fig. 2 lane 2 & Fig. 3 lane 2) though with a reduced intensity. Negative PCR amplification with FMDV amplimers both for the RNA Polymerase gene and the VP1 sequence was observed with RNA from sample D (Fig. 4) obtained from a one week old lamb. These results were confirmed by a serological test (CF test), inoculation to baby mice and infection of PK cell cultures. All 10 baby mice inoculated with the suspected material from each of the clinical samples A,B and C died within 48 hours, none of the mice inoculated with the material of sample D died. A complete CPE was observed within 24 hours in PK cell culture infected with the suspected material of samples

A,B and C. No CPE was observed in sample D. Complement Fixation test carried out with the field clinical material of sample A indicated relation of the suspected FMDV to the isolate 01 Geshur. Samples B and C obtained from different animals in the same outbreak were found by CF test to be related to the isolate 01 Dalton carried out following one passage on PK cell culture. Sample D was negative in the CF test.

DISCUSSION

The current availability of the PCR technique for the amplification of FMDV RNA (Laor et al, 1991 in preperation) and the novel and rapid method for poly A (+) RNA extraction using Dynabeads oligo (dT)25 allowed us to detect the presence of FMDV RNA in suspected biological material received from animals in the field. The combination of the two methods for RNA isolation and amplification enabled us to detect the presence of FMDV in a small epithelial tissue sample, shortly after arrival in the FMD Laboratory and without the need of passaging the virus in cell culture. The use of RNA Polymerase gene amplimers made possible the decision whether or not FMDV was present in the suspected biological material. Using specific amplimers located in the VP1 gene we could determine the relation of our FMDV isolate to type 01 as was later confirmed by the serological tests. CF was carried out on the suspected material in 2 out of the 4 samples immediatly as they arrived. In one case the sample was too small to be checked directly with the complement fixsation test, and in the other case the sample was anti complementary. A passage on PK cell culture before the CF test was inevitable in these cases.

The present method of molecular diagnosis of FMDV RNA in fild clinical material is rapid, accurate and prevents difficulty with the CF test and

the need to passage the virus in cell cultures. Further studies will allow direct sequencing of the VP1 gene of the new FMDV isolate to assess possible changes in the antigenic domains with the help of computer programs (Becker Y. to be published).

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Fig 1. RNA extracted by Dynabeads oligo (dT)25 from tongue epithelial tissue of an FMD suspected cow in the field (sample A). The RNA was amplified using the PCR technique, after cDNA synthesis, with specific primers for the RNA Polymerase gene and the VP1 area.

1. RNA extracted with 1.5 mg Dynabeads using Lysis/Binding buffer (as recommended by the manufacturer for solid tissue) and amplified with the PCR technique using oligonucleotides (# 7 & # 9) selected for the RNA polymerase gene.
2. RNA extracted with 450 µg Dynabeads using Lysis buffer (as recommended by the manufacturer for tissue culture cells) and amplified with the RNA polymerase gene amplimers (# 7 & # 9).
3. RNA from the sample used for lane 1 was amplified using specific amplimers for the VP1 area (# 2 & # 5) selected on the basis of the VP1 nucleotide sequence of O1 Geshur isolate.
4. RNA from the sample in lane 2 was amplified with amplimers # 2 & # 5.
5. Positive controle: RNA from BHK cells infected with FMDV O1 Geshur isolate. The amplimers for the RNA polymerase gene (# 7 & # 9) were used.
6. Negative control : no RNA or DNA present in the system.
7. DNA molecular weight size marker No. VI.

Fig. 2. RNA extracted with 1 mg Dynabeads oligo (dT)25 from tongue epithelial tissue of an FMD suspected cow in the field (sample B). The RNA was amplified with the PCR technique after cDNA synthesis using amplimers designated to amplify the RNA polymerase and the VP1 genes.

1. RNA extracted with 1 mg Dynabeads and amplified with the RNA polymerase gene amplimers (# 7 & # 9).
2. RNA amplified with the amplimers designated for amplification of the VP1 gene (# 2 & # 5).
3. Positive controle: RNA from BHK cells infected with FMDV O1 Geshur isolate. The amplimers for the RNA polymerase gene (# 7 & # 9) were used.
4. Negative control: : no RNA or DNA present in the system.
5. DNA molecular weight size marker No. VI.

Fig. 3. RNA extracted by Dynabeads oligo (dT)25 from foot skin epithelial tissue of an FMDV suspected sheep in the field (sample C). The extracted RNA was amplified, via cDNA synthesis, with the PCR technique using amplimers desgied to amplify the RNA polymerase and the VP1 genes .

1. The RNA was amplified with the RNA polymerase amplimers (# 7 & # 9).
2. The RNA was amplified with the VP1 amplimers (# 2 & # 5).
3. Positive control: RNA from BHK cells infected with FMDV O1 Geshur isolate. The amplimers for the RNA polymerase genes (# 7 & # 9) were used.
4. Negative control : No RNA or DNA present in the system.
5. DNA molecular weight size marker No. VI.

Fig. 4. RNA extracted by Dynabeads oligo (dT)25 from tongue epithelial tissue from an FMD suspected lamb (sample D). The RNA was amplified using the PCR technique after cDNA synthesis, with specific amplimers for the VP1 and the RNA polymerase genes.

1. The RNA was amplified with the VP1 area amplimers (# 2 & # 5). (a negative result)
2. The RNA was amplified with the RNA polymerase gene(# 7 & # 9). (a negative result)
3. Positive control:RNA from BHK cells infected with FMDV 01 Geshur isolate. The amplimers for the RNA Polymerase gene (# 7 & # 9) were used.
4. Negative control: no RNA or DNA present in the system.
5. DNA molecular weight size marker No. VI.

II Cloning of FMDV cDNA

The Israeli isolates O1 GESHUR and Asia-1 were sent from KVI to PIADC after receiving the necessary permit. The viruses were grown in bovine kidney cells to produce working stocks of viruses. Two passages were made and then grown in larger amounts on baby hamster kidney (BHK) cells.

A good yield of O1 GESHUR was obtained. The virus was concentrated with polyethylene glycol (PEG) and purified by ultracentrifugation on sucrose gradients. RNA was extracted from the purified virus with phenol-chloroform-isoamyl alcohol. One oligonucleotide primer residing in the 5' end of the P2 region close to the 3' end of VP1 was used (primer BNL172). The first cDNA sequence obtained by the use of the primer was composed of 153 bases. Sequencing of this DNA fragment revealed 76% homology to O1K FMDV isolate. A cDNA synthesis system was purchased (Riboclone-Promega) for the initial cDNA synthesis. For cloning of the P1 region of the genome (including VP1) the primer used in the initial sequencing was selected. Based on the lack of EcoRI sites in P1 region of O1K, 10-mer EcoRI linkers were used and the cDNA fragments were cloned in the KS plasmid (Stratagene, luc.) A similar approach was used for the cloning of Asia-1 cDNA.

The results are detailed in the following manuscript: Synthesis and cloning of FMDV cDNA from Asia 1 and O1 Geshur isolates.